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European Journal of Pharmacology 483 (2004) 229–232
www.elsevier.com/locate/ejphar

Short communication

Vinburnine decelerates [^{3}H]*N*-methylscopolamine binding to recombinant human muscarinic M_1–M_4 acetylcholine receptors

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Received 29 September 2003; received in revised form 22 October 2003; accepted 28 October 2003

Abstract

The kinetics of [^{3}H]*N*-methylscopolamine binding to membranes of Chinese hamster ovary (CHO) cells expressing muscarinic M_1–M_4 acetylcholine receptors was studied. [^{3}H]*N*-methylscopolamine dissociation was used for the "single-point" analysis of allosteric modulation by vinburnine (L-eburnamonine). [^{3}H]*N*-methylscopolamine dissociation was decelerated by vinburnine with EC_{50} values of 29.5, 4.1, 9.5 and 15.0 μM for muscarinic M_1–M_4 receptors, respectively. Acetylcholine doubled the EC_{50} of vinburnine for muscarinic M_3 receptors. These kinetic EC_{50} values correlated with equilibrium binding constants, supporting the ternary allosteric model. Vinburnine also decelerated the association of [^{3}H]*N*-methylscopolamine binding, resulting in opposite cooperativity for muscarinic M_1 and M_2 receptors.

Keywords: Muscarinic M_1–M_4 receptor; [^{3}H]*N*-methylscopolamine binding, kinetics of; Eburnamonine; Acetylcholine

1. Introduction

Muscarinic acetylcholine receptors play an important role in learning and memory. Five subtypes of muscarinic receptors have been cloned from human brain (Levey, 1996) and stably expressed in Chinese hamster ovary (CHO) cell cultures. A high-affinity muscarinic receptor antagonist, [^{3}H]*N*-methylscopolamine, is the most suitable radioligand to study muscarinic receptor binding. Several indole-type compounds affect [^{3}H]NMS binding and its rate of dissociation allosterically (Jakubík et al., 1997; Lazareno et al., 2000). Alcuronium, strychnine, vincamine, vinburnine (L-eburnamonine), indolocarbazoles (KT 5720), staurosporine and some steroidal derivatives bind to muscarinic receptors in micromolar concentrations and show cooperativity with the binding of agonists and antagonists (Jakubík et al., 1997; Lazareno et al., 2000, 2002; Proska and Tuček, 1994). The importance of the substituents of the indole ring system has been supported by the opposite cooperativity of vinburnine and vincamine with [^{3}H]NMS binding (Jakubík et al., 1997; Proska and Tuček, 1996).

There have been essentially two methods to study the allosteric effects of indole-type compounds on human muscarinic receptors. Long (22 h) incubation to obtain equilibrium [^{3}H]NMS binding and a ternary allosteric model led to the interpreration of enhancement or inhibition of [^{3}H]NMS binding with positive or negative cooperative binding interactions, respectively (Jakubík et al., 1997). For allosteric agents with "neutral cooperativity" a "single-point analysis" of [^{3}H]NMS dissociation is more suitable (Lazareno et al., 2000). The concentration dependence of allosteric modulation can thus be investigated for a fixed period of dissociation when dissociation is monophasic (Kostenis and Mohr, 1996). Recent results suggest divergent modes of action and/or more binding sites of allosteric agents (Lazareno et al., 2002; Tränkle and Mohr, 1997).

The aim of this study was to investigate the validity of the ternary allosteric model by using model-free kinetic methods and to explore the kinetic background of bidirectional allosteric interactions of vinburnine with different subtypes of muscarinic receptors. We applied "single-point" kinetic analysis of dissociation for vinburnine and demonstrate that the kinetic EC_{50} values agree with the binding constants according to the ternary allosteric model (Jakubík et al., 1997). The vasodilator vinburnine has optimal properties as an allosteric agent which binds to

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doi:10.1016/j.ejphar.2003.10.039

muscarinic receptor subtypes with relatively high affinity, selectivity and opposite cooperativity for [^{3}H]NMS binding. Both on and off rates of [^{3}H]NMS binding are decelerated by vinburnine and the balance of these processes determines whether there is positive or negative cooperativity.

2. Materials and methods

2.1. Materials

Human muscarinic M_1–M_4 receptors were expressed in CHO cell cultures and membranes were obtained from BioSignal-Packard. Membranes suspended in 50 mM Tris–HCl (pH = 7.4), 10% glycerol and 1% bovine serum albumin (0.6–4.3 mg protein/ml) were obtained in frozen state. [^{3}H]*N*-Methylscopolamine (83.5 Ci/mmol) was purchased from New England Nuclear (USA); (−)scopolcopolamine methylbromide and HEPES from Sigma; and guanosine-5′ -triphosphate·Na_3·$4H_2O$ (GTP) from Reanal (Budapest). Vinburnine was prepared at G. Richter.

2.2. Receptor binding

For dissociation studies, receptor suspensions were thawed and diluted by about ten times in incubation buffer containing 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$ at pH = 7.4. Muscarinic M_1, M_3 and M_4 receptors were incubated with 10–20 nM [^{3}H]NMS at 27 °C and M_2 receptors at 19 °C. Duplicate 20-μl aliquots were diluted into 2 ml of the incubation buffer, filtered immediately on GF/C filters under vacuum and rinsed with 3 × 3 ml cold washing buffer containing 10 mM sodium phosphate (pH = 7.4). After determination of total [^{3}H]NMS binding, 20-μl suspensions were diluted 100 times with incubation buffer containing 10 μM NMS to elicit dissociation. Dissociation was terminated after a fixed period via filtration. When used, acetylcholine (0.25 mM) and GTP (0.2 mM) were present both before and during dissociation. For association studies, receptor suspensions (2–12 μg protein/ml) were preincubated with or without 10–30 μM vinburnine and then with 0.3 nM [^{3}H]NMS. Duplicate 1-ml aliquots were filtered after up to 2 h of association.

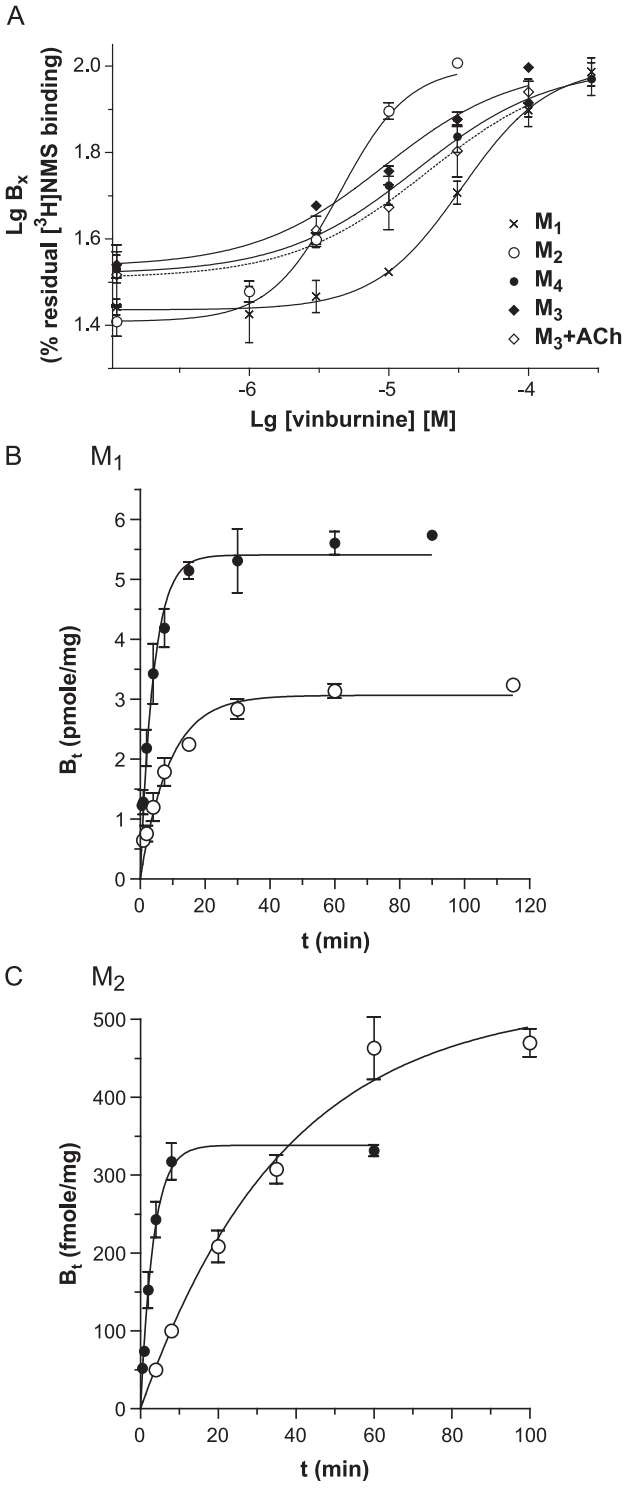

Fig. 1. The concentration dependence of the decelerating effects of vinburnine (A) after a constant period of dissociation of [^{3}H]NMS binding (10 min for muscarinic M_2 and 20 min for M_1, M_3 and M_4 receptors), and the effects of vinburnine on the association rate of [^{3}H]NMS binding to muscarinic M_1 (B) and M_2 (C) receptors. Data points are means ± S.E.M. of three to five experiments for A, three experiments for B and two representative experiments for C. Note that slope factors of the pooled data are 1.4 ± 0.2 and 1.7 ± 0.3 for muscarinic M_1 and M_2 receptors, respectively. The Hill parameters of the separate experiments are summarised in Table 1. Acetylcholine (0.25 mM ACh) decreased [^{3}H]NMS binding to muscarinic M_3 receptors to 89 ± 6%, shifted the response (dashed) curve of vinburnine and increased its EC_{50} to 19.4 ± 2.8 μM with a slope factor of 1.1 ± 0.2 (mean ± S.E.M., n = 3). B_t is specifically bound [^{3}H]NMS/mg protein as a function of the time (t) of association. Receptor suspensions (2–12 μg protein/ml) were preincubated in the absence (control: •) and presence of vinburnine (o) for 20 min and then with 0.3 nM [^{3}H]NMS. Vinburnine concentrations were 30 μM for muscarinic M_1 and 50 μM for M_2 receptors. Temperature of incubation: 27 °C for muscarinic M_1 and 19 °C for M_2 receptors.

2.3. Data analysis

Nonspecific [^{3}H]NMS binding in the presence of 5 μM atropine was subtracted from total binding. Residual binding after up to 20 min of dissociation (B_t) was divided by specific binding at $t=0$ (B_0) and expressed in %. Data were plotted on a semilogarithmic plot as a function of time. Linear regression was performed via GraphPad Prism (San Diego, USA). Rate constants and half-lives of [^{3}H]NMS dissociation were calculated from the slope of the regression lines.

A dissociation period equivalent to one to two times the half-life was fixed. Residual binding (B_x) was divided by control residual binding in the absence of vinburnine (B_c) and expressed in %. Fig. 1A gives log B_x values, the antilog of which represents % residual binding after a fixed time of dissociation. Values for log B_x were plotted as a function of vinburnine concentration. Data were fitted to the Hill equation with variable slopes by using Prism. Half-maximal deceleration of dissociation (EC_{50}) and slope values were determined from the fit.

Apparent rate constants of association (k_{app}) were determined by fitting an equation with one exponential by using Prism. Association rate constants (k_{on}) were calculated from Eq. (1):

$$k_{app} = ck_{on} + k_{off} \quad (1)$$

where c is the concentration of [^{3}H]NMS. Dissociation rate constants (k_{off}) were determined from independent experiments.

3. Results

The time dependence of dissociation of [^{3}H]NMS binding was examined at 27 °C in preliminary experiments. Dissociation was rather slow and monophasic up to 20 min of dissociation (data not shown). The effects of vinburnine were examined for this period of dissociation for muscarinic M_1, M_3 and M_4 receptors. Since [^{3}H]NMS dissociation was rather rapid for muscarinic M_2 receptors, the incubation temperature was decreased to 19 °C and the effects of vinburnine were then examined for 10 min of dissociation. There was a concentration-dependent deceleration of [^{3}H]NMS dissociation for all muscarinic M_1–M_4 receptors (Fig. 1A). Deceleration approached a maximum at complete inhibition (log 100% = 2 in Fig. 1A). Fitting the Hill equation to the data for vinburnine resulted in the response curves shown in Fig. 1A. Differences in control binding (in the absence of vinburnine, on the ordinate) are arbitrary, due to the different extent of dissociation. The means of the computer-fitted EC_{50} and slope values are summarized in Table 1. The slope values for vinburnine were higher than unity for muscarinic M_1 and M_2 receptors and close to unity for M_3 and M_4 receptors. The effect of acetylcholine on [^{3}H]NMS binding to muscarinic M_3 receptors with neutral cooperativity ($\alpha \approx 1$, Table 1) was examined in the presence of 0.2 mM GTP to eliminate high-affinity acetylcholine binding. Acetylcholine (0.25 mM) decreased [^{3}H]NMS binding to 89 ± 6% and significantly increased the EC_{50} of vinburnine from 9.5 ± 1.1 μM to 19.4 ± 2.8 μM (means ± S.E.M., $n=3$, $P<0.05$, Fig. 1A).

Table 1
The effect of vinburnine on the dissociation of [^{3}H]NMS binding to recombinant muscarinic receptors

Muscarinic receptor subtype	Dissociation of [^{3}H]NMS binding			Modulation of equilibrium [^{3}H]NMS binding[a]
	k_{off}[b] (min^{-1})	Allosteric deceleration		Cooperativity with vinburnine
		EC_{50} (μM)	Slope	$\alpha^c \times K_d$ (μM)
M_1	0.067 ± 0.002	29.5 ± 2.5	1.3 ± 0.1[d]	2.7 × 7.5 = 20.3
M_2	0.135 ± 0.006	4.1 ± 0.5	1.4 ± 0.2[d]	0.12 × 68.9 = 8.3
M_3	0.041 ± 0.002	9.5 ± 1.1	1.0 ± 0.1	0.99 × 6.7 = 6.6
M_4	0.068 ± 0.003	15.0 ± 3.0	0.9 ± 0.1	0.27 × 23.7 = 6.4

Data are means ± S.E.M. of three to five individually fitted experiments.
[a] From Jakubík et al., 1997.
[b] Rate constants were determined at 27 °C except for muscarinic M_2 receptors (19 °C).
[c] Coefficient of cooperativity with [^{3}H]NMS binding. Cooperativity is positive if $\alpha<1$, while it is negative if $\alpha>1$.
[d] $P<0.1$, significantly different from unity in Student's t test.

Association rates of [^{3}H]NMS binding were also examined for muscarinic M_1 and M_2 receptors with atypical response curves. Fig. 1B and C confirms the opposite effects of vinburnine on [^{3}H]NMS binding equilibrium (Jakubík et al., 1997). Association was apparently monophasic (Fig. 1B and C). For muscarinic M_1 receptors, 30 μM vinburnine decreased the rate constant of control association (k_{on}) from 0.60 ± 0.07 $(min{\cdot}nM)^{-1}$ to 0.25 ± 0.07 $(min{\cdot}nM)^{-1}$ significantly ($P<0.02$, $n=4$). For muscarinic M_2 receptors, control association with a k_{on} value of 0.477 ± 0.100 $(min{\cdot}nM)^{-1}$ (mean ± S.E.M. of five experiments) was not decelerated by 10 μM vinburnine significantly (data not shown). Therefore, association was also examined in the presence of 50 μM vinburnine (Fig. 1C), which inhibits [^{3}H]NMS dissociation completely. This vinburnine concentration decelerated [^{3}H]NMS association to 24%: k_{on} = 0.117 ± 0.031 $(min{\cdot}nM)^{-1}$ (significantly less than control, $P<0.05$, $n=3$).

4. Discussion

Several allosteric agents of muscarinic receptors, such as gallamine and alcuronium, decelerate both on and off rates of [^{3}H]NMS binding (Schröter et al., 2000). However, the dissociation of [^{3}H]NMS binding can be modulated in either direction and to a different maximal extent (Lazareno et al., 2000, 2002). The decelerated dissociation of [^{3}H]NMS

enabled us to use the "single-point" kinetic analysis. Vinburnine caused complete deceleration of [^{3}H]NMS binding to all subtypes of human muscarinic receptors. The kinetic EC_{50} values of vinburnine correlated with the apparent equilibrium binding constant αK_d rather than with the K_d itself (Table 1). Here, α is the coefficient of cooperativity between the antagonist and the allosteric agent as determined for equilibrium [^{3}H]NMS binding (Jakubík et al., 1997). It can be envisaged that for allosteric agents having neutral cooperativity with [^{3}H]NMS, "single point" kinetic analysis might reveal cooperativity with muscarinic agonists as well. Indeed, for muscarinic M_3 receptors, acetylcholine increased the EC_{50} of vinburnine 2.0-fold, which corresponds to $\beta = 1.96$ (Jakubík et al., 1997), reflecting negative cooperativity between acetylcholine and vinburnine. Thus, for allosteric agents in neutral cooperativity with [^{3}H]NMS binding, cooperativity with muscarinic agonists can be demonstrated via the kinetic EC_{50} value. Consequently, the ternary allosteric model seems to be valid for vinburnine.

Bidirectional cooperativity between vinburnine and [^{3}H]NMS binding can be attributed to one-directional kinetic modulation of [^{3}H]NMS binding. For muscarinic M_1 receptors, deceleration of association outweighed that of dissociation, resulting in negative cooperativity. In contrast, predominant deceleration of dissociation resulted in positive cooperativity for muscarinic M_2 receptors. This can be reconciled with the hypothetical steric hindrance of orthosteric binding sites by allosteric agents (Tuček and Proska, 1995). Agonists bind to orthosteric sites competitively with antagonists. Therefore, it is probable that the bidirectional cooperativity of muscarinic agonists with allosteric agents (Jakubík et al., 1997) can be attributed to an analogous (steric) mechanism of deceleration.

In conclusion, vinburnine is a relatively high-affinity, subtype-selective allosteric agent of human recombinant muscarinic receptors, and decelerates the kinetics of [^{3}H]NMS binding. The ternary allosteric model seems to be valid for vinburnine. The opposite cooperativity of vinburnine can be reconciled with its decelerating effects for both on and off rates of [^{3}H]NMS binding, the balance of which varies for different subtypes of muscarinic receptors. The orthosteric site seems to be sterically hindered by vinburnine (Tuček and Proska, 1995).

Acknowledgements

The authors thank Drs. Sándor Farkas and Ferenc Auth (G. Richter) for stimulating discussions.

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